Potent Inhibition of Human Phosphodiesterase-5 by Icariin Derivatives

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Plant extracts traditionally used for male impotence (*Tribulus terrestris, Ferula hermonis, Epimedium brevicornum, Cinnamomum cassia*), and the individual compounds cinnamaldehyde, ferutinin, and icariin, were screened against phosphodiesterase-5A1 (PDE5A1) activity. Human recombinant PDE5A1 was used as the enzyme source. Only *E. brevicornum* extract (80% inhibition at 50 μg/mL) and its active principle icariin (**1**) (IC_{50} 5.9 μM) were active. To improve its inhibitory activity, **1** was subjected to various structural modifications. Thus, 3,7-bis(2-hydroxyethyl)icaritin (**5**), where both sugars in **1** were replaced with hydroxyethyl residues, potently inhibited PDE5A1 with an IC_{50} very close to that of sildenafil (IC_{50} 75 vs 74 nM). Thus, **5** was 80 times more potent than **1**, and its selectivity versus phosphodiesterase-6 (PDE6) and cyclic adenosine monophosphate-phosphodiesterase (cAMP-PDE) was much higher in comparison with sildenafil. The improved pharmacodynamic profile and lack of cytotoxicity on human fibroblasts make compound **5** a promising candidate for further development.

The inability to achieve or maintain an erection sufficient for satisfactory sexual function is an increasing problem with a considerable impact on interpersonal relationships and quality of life for men.[1] During erection, nitric oxide is released from the axon terminals of the parasympathetic nerves and diffuses into smooth muscle cells of the arterial walls of the corpus cavernosum. The consequent activation of guanyl cyclase, converting guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP), causes smooth muscle relaxation, leading to dilation and increased influx of blood into the penile tissue. The trapping of blood in the penis results in an erection.[2] Selective inhibitors of cGMP-phosphodiesterase-5 (PDE5) such as sildenafil (Viagra), tadalafil, and vardenafil are currently used for erectile dysfunction (ED). However, several adverse effects have been recorded in clinical trials, including priapism and visual disturbances.[3] Furthermore, therapy with PDE5 inhibitors is cost-effective. Thus, the search for new compounds of this type for drug development could be worthwhile. A variety of natural plant products, including berberine, forskolin, papaverine, and yohimbine, are claimed to be useful for improving sexual performance. Extracts from *Lepidium meyenii* Walp. (maca), *Panax ginseng* C.A. Meyer, *Ginkgo biloba* L., *Ferula hermonis* Boiss., and many other herbal remedies, alone or in combination, have been promoted for the treatment of sexual problems.[4,5] With the aim of looking for new leads for selective PDE5 inhibitors, plant extracts and their putative active principles were selected for screening against human PDE5 activity in vitro. Our attention focused on *Tribulus terrestris* L., *Ferula hermonis*, *Epimedium brevicornum* Maxim., and *Cinnamomum cassia* L., since these extracts are claimed traditionally to improve sexual performance. *T. terrestris* caused vasodilating and antihypertensive effects in rats[6] and a pro-erectile effect on the rabbit corpus cavernosum;[7] *F. hermonis* has been studied for its effects on sexual behavior in male and female rats;[8,9] *C. cassia* and "Epimedii Herba" are components of Chinese herbal products patented for the treatment of sexual dysfunction.[10−14] "Epimedii herba" is the common name for the dried aerial parts of *E. brevicornum*, *E. sagittatum* Maxim., or *E. koreanum* Nakai, collected in the summer.[15,16] Among the extracts, only "Epimedii Herba" was active against PDE5A1, for which the presence of icariin (**1**), the major pharmacologically active constituent,[17−19] was considered a lead compound for chemical modifications in order to improve inhibitory activity. Modifications applied at the hydroxyl groups at C-3, C-7, and C-8 included partial or complete removal of the sugar moieties, partial or complete sugar replacement with a hydroxyethyl residue, and cyclization of the prenyl group (Scheme 1). All compounds produced (**1**−**6**) were tested for PDE5A1 activity. Also, selectivity versus human retina PDE6C and human platelet cAMP-PDE, and cytotoxicity on human fibroblasts were investigated.

Results and Discussion

The activity of plant extracts and individual compounds against human recombinant PDE5A1 is shown in Figure S1 (Supporting Information). Cinnamaldehyde, icariin (**1**), and ferutinin were considered as the putative active principles of *C. cassia*, *E. brevicornum*, and *F. hermonis*, respectively, since the compounds represent the most abundant secondary metabolites of those species. Only *E. brevicornum* and icariin (**1**) strongly inhibited PDE5A1 (−80% and −72%, respectively), whereas the other test materials were much less active (−15 to −23%). Inhibition by cinnamaldehyde (−16%) and ferutinin (−7%) was not significant. The medicinal plants tested in the present study had a reputation for aphrodisiac effects and therefore represented the start of a screening program to search for compounds to be developed as a new natural drug alternative to sildenafil. The observation that only *E. brevicornum* and its active principle **1** inhibited PDE5 in a significant manner, in agreement with previous results,[17−20] suggests that the other plant extracts may interfere with erectile function through mechanisms other than PDE5 inhibition.

Compound **1** was a good PDE5 inhibitor (IC_{50} of 5.9 μM), but required improvement in order to have equivalent potency to sildenafil, which gave an IC_{50} of 75 nM. The inhibitory effects of icariin derivatives **2**−**6** on PDE5A1 is shown in Table 1. Since aglycons might be expected to possess higher activity than the corresponding glycosides, the first general structural modification to **1** was the removal of one or both of the sugar moieties at the hydroxyl groups at positions C-3 and C-7 of the flavone scaffold. Enzymatic hydrolysis of **1** with cellulase and naringinase allowed the partial or total removal of the sugar moieties, respectively,

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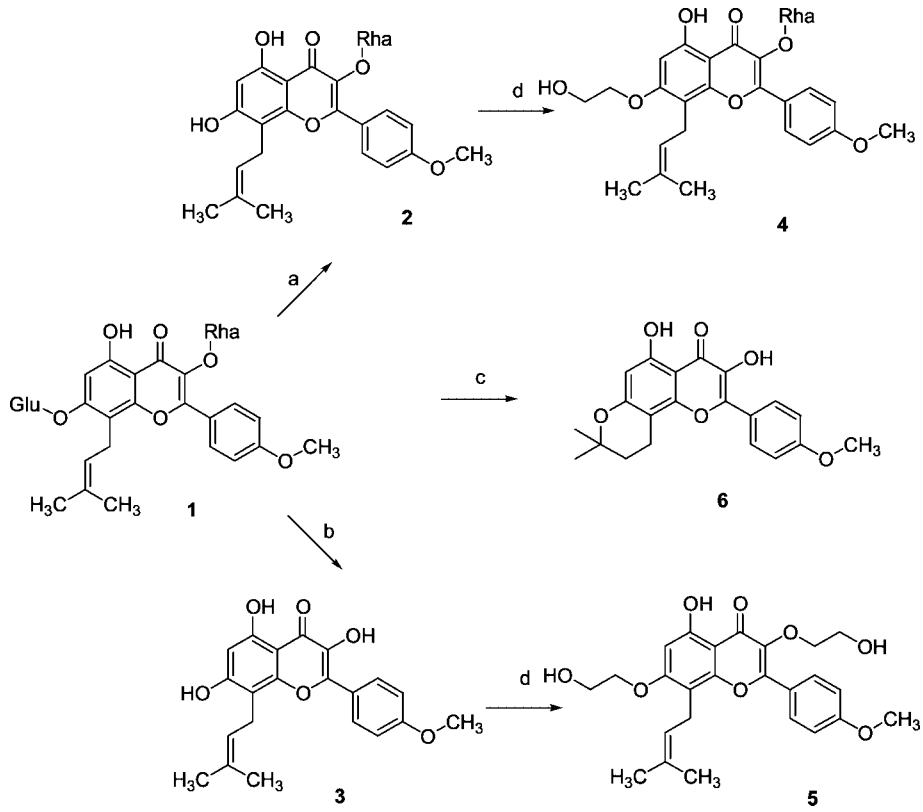
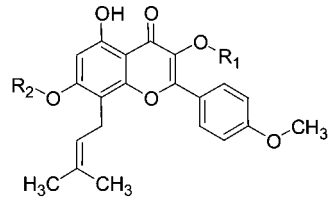
Scheme 1. Outline of the Synthetic Route Followed for the Synthesis of Icariin (**1**) Derivatives[a]

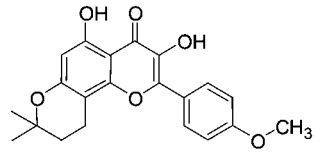


[a] Reagents and conditions: (a) cellulase, 37 °C, Na acetate pH = 5 buffered hydroalcoholic solution, 6 days; (b) naringinase, 37 °C, Na acetate pH = 5 buffered hydroalcoholic solution, 11 days; (c) H_2SO_4, dioxane, reflux, 24 h; (d) 2-bromoethanol, K_2CO_3, acetone, reflux, 8 h.

Chart 1



	R_1	R_2	
1	Rha	Glc	icariin
2	Rha	H	icariside II
3	H	H	icaritin
4	Rha	$-CH_2-CH_2-OH$	7-(2-hydroxyethyl)-3-*O*-rhamnosylicariin
5	$-CH_2-CH_2-OH$	$-CH_2-CH_2-OH$	3,7-bis-(2-hydroxyethyl)-icaritin



β-anhydroicaritin (**6**)

affording the known compounds **2** and **3**. Indeed, the removal of the glucose at the hydroxyl group in C-7, thus furnishing icariside II (**2**), improved drastically the enzyme inhibition, attaining an IC_{50} value on the nanomolar order (IC_{50} 156 nM). Conversely, icaritin (**3**), where both sugars were removed, was only around 3-fold more potent than **1** (IC_{50} 2.2 μM).

To investigate if the prenyl moiety is essential for enzyme inhibition activity, β-anhydroicaritin (**6**) was tested. The cyclization

Table 1. IC_{50} Values of Icariin Derivatives and Sildenafil on Human PDE5A1

compound	PDE5A1 (IC_{50} μM ± SD)
1	5.9 ± 1.1
2	0.16 ± 0.02
3	2.2 ± 0.09
4	0.36 ± 0.06
5	0.074 ± 0.007
6	45.5 ± 4.6
sildenafil	0.075 ± 0.004

led to a dramatic drop in inhibitory activity. The IC_{50} value for **6** was 45.5 μM, indicating that a free prenyl group at position C-8 is important for enzyme inhibition. To confirm that the prenyl group is required for enzyme inhibition, the 8-prenyl derivatives of naringenin (8-PN), quercetin (8-PQ), and apigenin (8-PA) were tested and their activity compared to that of the corresponding free flavonoid. As shown in Figure S2 (Supporting Information), all prenylflavonoids inhibited PDE5A1 with the following order of potency: 8-PQ (IC_{50} 0.70 ± 0.10 μM) > 8-PA (IC_{50} 1.29 ± 0.11 μM) ≫ 8-PN (IC_{50} 16.23 ± 1.16 μM). Quercetin, apigenin, and naringenin (10 μM) showed 23%, 12%, and 6% inhibition, respectively, much lower than the corresponding prenyl derivatives. Data from the literature confirm the importance of the prenyl group: sophoflavescenol, a prenylated flavonol from *Sophora flavescens* Ait. (Leguminosae), and osthole, a prenyl coumarin from *Angelica pubescens* Maxim., are two additional examples of PDE-5 inhibitors in the class of prenylated phenolic compounds.[21,22]

The last modification to **1** was the replacement of one or both sugar moieties with the hydroxyethyl side chain, representing a simplification of the sugar residue. The substitution for Glc by a hydroxyethyl group at C-7 gave 7-(2-hydroxyethyl)-3-*O*-rhamnosylicariin (**4**), which was less potent than **2** (IC_{50} 363 vs 156 nM, respectively). When both hydroxyls at C-3 and C-7 were derivatized with hydroxyethyl moieties, as in 3,7-bis(2-hydroxyethyl)icaritin (**5**), PDE5A1 was potently inhibited, with an IC_{50} of 74 nM, almost identical to that of sildenafil (75 nM).

The selectivity against human PDE5A1 was investigated by testing all compounds against human PDE6C, at concentrations 10-fold higher than their PDE5A1 IC_{50} values. Compounds **1** and **4** inhibited PDE6 activity (45% and 86%, respectively), while **2**, **3**, **5**, and **6** were inactive. For **5**, the best PDE5 inhibitor among the icariin derivatives tested, concentration inhibition curves for PDE6 and cAMP-PDE were performed. The results were compared with those obtained for sildenafil (Table 2). The PDE6C/PDE5 IC_{50} ratio was 418 for **5** and 2.2 for sildenafil, while the cAMP-PDE/PDE5 IC_{50} ratio was 1300 for **5** and 367 for sildenafil. These results indicate that the selectivity of **5** for PDE5 was improved with respect to that of sildenafil. Compound **5** was not cytotoxic for human fibroblasts even at the highest concentration tested (100 μM). Thus, the inhibitory potency of **5** was 80-fold higher than that of the parent compound icariin (**1**). Its selectivity and lack of cytotoxicity make **5** a candidate worthy of further study.

Experimental Section

General Experimental Procedures. Melting points were determined in open glass capillaries using a Büchi apparatus and are uncorrected. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian VXR 200 or Varian VXR 300 spectrometer equipped with VNMR software. Chemical shifts (δ) are reported in ppm with tetramethylsilane (TMS) as the internal standard, and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), dd (double doublet), dt (double triplet), m (multiplet), or br (broad). ESIMS were obtained on a Finnigan MAT LCQ ion trap mass spectrometer or Waters Micromass ZQ 4000 apparatus equipped with a Microsoft Windows NT data system and an ESI interface. HPLC-MS analysis was carried out with a Waters 600 MS liquid chromatograph equipped with an Agilent Zorbax SB C_{18} column (4.6 mm × 2.5 cm) held at 35 °C and a Waters 486 tunable detector set at 289 nm. Analytical conditions were as follows: elution gradient 0.01% trifluoroacetic acid in CH_3CN (A) and 0.01% trifluoroacetic acid (v/v) in water (B) eluting in gradient mode starting from 10% (A) up to 60% (A) in 40 min at a flow rate of 1.0 mL/min.

HPLC-UV analysis was carried out with a Kontron 325 pump/system controller equipped with a Merck-Hitachi UV−vis detector set to 278 nm. The analyses were performed on Phenomenex Luna RP C_{18} (3 μm, 4.6 mm × 1.5 cm) columns. Analytical conditions were as follows: elution gradient CH_3CN (A) and 0.01% trifluoroacetic acid (v/v) in water (B) according to the following profile: 0−60 min, 15−100% A, 85−0% B; flow rate 1.0 mL/min.

All solvents and reagents were obtained from commercial sources and used without further purification unless otherwise noted. Reaction courses and product mixtures were routinely monitored by TLC and HPLC. TLC was carried out on precoated silica gel F_{254} (Merck) plates or on silica gel 60 (Merck) plates (visualizing developed chromatograms by spraying plates with 20% CH_2O/H_2SO_4 followed by heating at 100 °C for 3 min). Column chromatography was carried out with silica gel (Kieselgel 40, 0.040−0.063 mm; Merck) using the flash technique. For the semisynthetic derivatives, yields are reported after chromatographic purification and crystallization.

Dulbecco's modified Eagle's medium, trypsin, protease inhibitors, naringinase (from *Penicillium decumbens*, 596 units/g solid; β-glucosidase activity: 69 units/g solid), and all chemical reagents for cell culture were purchased from Sigma Aldrich (Milan, Italy). Cellulase (from *Aspergillus niger*) was from Fluka (Milan, Italy). Penicillin, streptomycin, and L-glutamine were from GIBCO (Grand Island, NY); fetal calf serum was provided by Mascia Brunelli SpA (Milan, Italy). The COS-7 cell line was purchased from ATCC (Manassas, VA). Superfect reagent for transient transfections was obtained from Qiagen GmbH (Hilden, Germany). The expression plasmid pcDNA3 containing the full-length cDNA of PDE5A1 was a kind gift of Prof. C. S. Lin (Department of Urology, University of California, San Francisco, CA). Human recombinant PDE6C, cloned from the human retina and expressed in *S. frugiperda* insect cells using a baculovirus expression system, was purchased from Scottish Biomedical (Glasgow, UK). [^{3}H]-cGMP and [^{3}H]-cAMP were from Amersham Pharmacia Biotech (Amersham Place, Little Chalfont, Buckinghamshire, UK). DEAE-Sephadex A25 was from Pharmacia (Uppsala, Sweden). cGMP, cAMP, AMP, and *Crotalus adamanteus* snake venom were purchased from Sigma Aldrich. Sildenafil was provided by Sequoia Research Products (Oxford, UK). Cinnamaldehyde and ferutinin were supplied by Indena Spa (Milan, Italy). 8-Prenylnaringenin, 8-prenylquercetin, and 8-prenylapigenin (purity >98%) were donated by Prof. Giovanni Appendino (Università del Piemonte Orientale, Italy).

Plant Material. *T. terrestris* L. dried extract (44% furostanolic saponins) was from Farmbio Ltd. (Sofia, Bulgaria); the ethanolic extract of the aerial parts of *E. brevicornum* Maxim. (20.9% icariin) was from Chengdu Wagott Natural Products Co. Ltd., Xian City, People's Republic of China. The root methanolic extract from *F. hermonis* Boiss. (26.3% ferutinin) and *C. cassia* L. oil extract (73.4% cinnamaldehyde) were supplied by Indena Spa (Milan, Italy). Plant material was identified against a crude drug standard and/or authoritative literature source by a suitable qualified person. A voucher of each plant is kept at the botanical laboratory of the company. Extracts were quantified by HPLC, and the chromatographic profiles are shown in Figures S3−S6 (Supporting Information).

Extraction and Isolation of Icariin (1). A dried extract of *E. sagittatum* as a greenish-brown residue (4 g) was dissolved in a mixture of CH_3OH/H_2O (1:1) (200 mL). The solution was stirred for 20 min and then washed with CH_2Cl_2 (3 × 80 mL). Methanol was evaporated under vacuum, and the remaining aqueous solution was diluted with water to 400 mL. The solution was extracted with EtOAc (5 × 400 mL), and the organic phase was taken to dryness. The extract was resuspended with CH_2Cl_2 (200 mL) and filtered under vacuum to yield 1.08 g of extract (A), from which icariin (**1**) was purified by precipitation with methanol (50 mL) as a yellow powder (purity 95.3%) (530 mg; 13% yield on the dry extract); mp 224−226 °C;[22] ^{1}H NMR (DMSO-d_6, 300 MHz, 30 °C) δ 0.80 (3H, d, J = 5.4 Hz, rha CH_3), 1.60 (3H, s, CH_3-14), 1.70 (3H, s, CH_3-15), 3.05−3.20 (4H, m, H-11 and sugar protons), 3.40−3.80 (7H, m, sugar protons), 3.87 (3H, s, OCH_3), 4.00 (1H, m, sugar proton), 4.55−4.78 (3H, m, OH), 4.85−5.22 (6H, m, sugar protons and OH), 5.30 (1H, t, J = 6.9 Hz, H-12), 6.60 (s, 1H, H-6), 7.15 (2H, d, J = 8.4 Hz, H-3′, H-5′), 7.85 (2H, d, J = 8.4

Table 2. IC_{50} Values of **5** and Sildenafil on Human PDE6 and cAMP-PDE

compound	PDE6C (IC_{50} μM ± SD)	PDE6C/PDE5A1 (IC_{50} ratio)	cAMP-PDE (IC_{50} μM ± SD)	cAMP-PDE/PDE5A1 (IC_{50} ratio)
5	30.9 ± 2.6	418	96.3 ± 12.9	1301
sildenafil	0.16 ± 0.007	2.2	27.5 ± 5.3	367

Hz, H-2′, H-6′), 12.60 (s, 1H, OH-5); ESIMS (positive-ion mode) *m/z* 677 $[M + H]^+$, 699 $[M + Na]^+$.

Preparation of Icariside II (2). A solution of **1** (500 mg) in DMSO (1 mL) was added dropwise for 48 h to a Na acetate-buffered hydroalcoholic solution at 37 °C (0.25 M, pH 5.0, in EtOH/H_2O, 30:70) (50 mL) containing cellulase (210 mg). The suspension obtained was stirred at 37 °C for 4 days. Then, a further amount of cellulase (100 mg) was added, and the mixture was stirred under the same conditions for a further 2 days. EtOH was then removed under vacuum and the residue was diluted to 200 mL with H_2O and extracted with EtOAc (3 × 200 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford **2** (290 mg); yield 76%; mp 208−210 °C;[23] ^{1}H NMR (DMSO-d_6, 300 MHz, 30 °C) δ 0.90 (3H, s, rha CH_3), 1.82 (3H, s, CH_3-14), 1.87 (s, 3H, CH_3-15), 3.05−3.60 (4H, m, rha protons, H-11), 3.85 (3H, s, OCH_3), 4.22−4.24 (1H, m, rha proton), 4.55−4.80 (3H, m, sugar OH), 4.90 (1H, m, rha proton), 5.20 (1H, t, J = 6.8 Hz, H-12), 5.52 (1H, d, J = 1.5 Hz, rha proton), 6.37 (1H, s, H-6), 7.15 (2H, d, J = 8.4 Hz, H-3′, H-5′), 7.83 (2H, d, J = 8.4 Hz, H-2′, H-6′), 10.60 (1H, s, OH-7), 12.80 (1H, s, OH-5); ESIMS (positive-ion mode) *m/z* 537 $[M + Na]^+$.

Preparation of Icaritin (3). A solution of icariin (**1**) (526 mg) in DMSO (1 mL) was added dropwise for 72 h to a Na acetate-buffered hydroalcoholic solution at 37 °C (0.25 M, pH 5.0, in EtOH/H_2O, 30:70) (50 mL) containing naringinase (207 mg). The obtained suspension was allowed to stir at 37 °C for 7 days. Then, a further amount of naringinase (97 mg) was added and the mixture was stirred under the same conditions for a further day. EtOH was removed by evaporation and the aqueous suspension was filtered under vacuum and dried. The residue obtained was washed with H_2O and dried to give icaritin (**3**, 290 mg; purity 95%) as a yellow powder. The mother liquors were diluted with H_2O and extracted with EtOAc (2 × 200 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford an additional amount of **3** (20 mg); quantitative yield, mp 232−233 °C;[24] ^{1}H NMR ($CDCl_3$, 300 MHz, 30 °C) δ 1.78 (3H, s, CH_3-14), 1.87 (3H, s, CH_3-15), 2.70 (2H, s, OH), 3.61 (2H, d, J = 6.8 Hz, H-11), 3.89 (3H, s, OCH_3), 5.36 (1H, t, J = 6.8 Hz, H-12), 6.32 (1H, s, H-6), 7.04 (2H, d, J = 8.4 Hz, H-3′, H-5′), 8.16 (2H, d, J = 8.4 Hz, H-2′, H-6′); ESIMS (positive-ion mode) *m/z* 369 $[M + H]^+$.

Preparation of 7-(2-Hydroxyethyl)-3-*O*-rhamnosylicariin (4). A stirred suspension of **2** (200 mg, 0.39 mmol), 2-bromoethanol (50 mg, 0.43 mmol), and anhydrous K_2CO_3 (60 mg, 0.43 mmol) in dry acetone (15 mL) was refluxed for 8 h. The hot reaction mixture was filtered, and the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel (EtOAc/CH_3OH, 9.5:0.5) to give a yellow crystalline compound (**4**, 112 mg; purity 93.0%); 55% yield; mp 194−196 °C (EtOH); ^{1}H NMR (acetone-d_6 + D_2O, 300 MHz, 30 °C) δ 0.88 (3H, d, J = 5.4 Hz, rha CH_3), 1.64 (3H, s, CH_3-14), 1.75 (3H, s, CH_3-15), 3.2−3.8 (3H, m, rha protons), 3.7 (2H, d, J = 8.7, H-11), 3.91 (3H, s, OCH_3), 4.21−4.24 (2H, m, OCH_2O), 4.40 (2H, t, J = 6.0 Hz, CH_2OH), 4.22−4.24 (1H, m, rha proton), 5.25 (1H t, J = 6.9 Hz, H-12), 5.52 (1H, d, J = 1.5 Hz, rha proton), 6.50 (1H, s, H-6), 7.14 (2H, d, J = 6.9 Hz, H-3′, H-5′), 7.96 (2H, d, J = 6.9 Hz, H-2′, H-6′); ESIMS (positive-ion mode) *m/z* 581 $[M + Na]^+$; *anal*. C 62.31%, H 6.18%, calcd for $C_{29}H_{34}O_{11}$, C 62.36%, H 6.14%.

Preparation of 3,7-Bis(2-hydroxyethyl)icaritin (5). A stirred suspension of **3** (250 mg, 0.7 mmol), 2-bromoethanol (210 mg, 1.7 mmol), and anhydrous K_2CO_3 (240 mg, 1.7 mmol) in dry acetone (75 mL) was refluxed for 8 h. The hot reaction mixture was filtered, and the solvent was evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2/acetone, 9:1) and crystallized from EtOH to give the desired compound as a yellow crystalline powder (70 mg, purity 96.0%); 20.2% yield; mp 152−153 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, 30 °C) δ 1.77 (3H, s, CH_3-14), 1.87 (3H, s, CH_3-15), 3.61 (2H, d, J = 6.8 Hz, H-11), 3.78−3.83 (2H, m, 7-OCH_2O), 3.90 (3H, s, OCH_3), 3.95−4.05 (4H, m, CH_2OH), 4.15−4.22 (2H, m, OCH_2O-3), 5.19 (1H, t, J = 6.8 Hz, H-12), 6.32 (1H, s, H-6), 7.04 (2H, d, J = 8.4 Hz, H-3′, H-5′), 8.16 (2H, d, J = 8.4 Hz, H-2′, H-6′); ESIMS (positive-ion mode) *m/z* 479 $[M + Na]^+$; *anal*. C 65.82%, H 6.22%, calcd for $C_{25}H_{28}O_8$, C 65.78%, H 6.18%.

Preparation of β-Anhydroicaritin (6). A solution of extract A, used in the isolation of **1** (200 mg) in dioxane (25 mL), was added to 1 M H_2SO_4 (12.5 mL) and refluxed for 24 h. After cooling, the reaction mixture was adjusted to pH 7−8 with $NaHCO_3$ and extracted with EtOAc (3 × 50 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated under vacuum to afford β-anhydroicaritin as a yellow powder (purity 96.5%; 105 mg, 23% yield of the dry extract);[25] ^{1}H NMR (DMSO-d_6, 30 °C) δ 1.40 (6H, s, 14, CH_3-15), 1.90 (2H, t, H-12), 2.85 (2H t, H-11), 3.90 (3H, s, OCH_3), 6.20 (1H, s, H-6), 7.15 (2H, d, J = 8.9 Hz, H-3′, H-5′), 8.20 (2H, d, J = 8.9 Hz, H-2′, H-6′), 9.57 (1H, s, OH-3), 12.20 (1H, s, OH-5); ESIMS (positive-ion mode) *m/z* 369 $[M + H]^+$.

Human Recombinant PDE5A1 Expression. Human recombinant PDE5A1 was prepared by expression of the full-length cDNA of PDE5A1 into COS-7 cells, as previously described.[26]

PDE5A1 and PDE6C Enzyme Assays. PDE5A1 activity was determined according to the method of Kincaid and Manganiello[27] with minor modifications.[28] Screening of plant extracts was performed at 50 μg/mL, whereas the individual compounds were tested at 10 μM. PDE6C activity was evaluated under the same conditions used for PDE5A1 activity, with 0.5 U enzyme/sample being used. Screening of the individual compounds against PDE6C activity was performed at concentrations 10-fold higher than each IC_{50} obtained against PDE5A1. IC_{50} values were calculated using Graph Pad Prism 4 for sigmoidal curves. Sildenafil was used as reference compound. Each result is the mean ± SD of at least two experiments in triplicate.

Platelet Homogenate Preparation and Assay for cAMP-PDE Activity. The blood fraction enriched in platelets, obtained from healthy volunteers, was submitted to two centrifugations at 160*g* for 10 min at room temperature. The pellet was removed, and platelet-rich plasma (PRP) was centrifuged at 1000*g* for 15 min. The resulting pellet was suspended in 10 mM Tris/HCl, pH 7.4 (2/5 of the initial volume). The suspension was centrifuged at 1000*g* for 15 min and the pellet suspended in the Tris/HCl buffer, pH 7.4 (1/12 of the initial volume). All these steps were performed at 4 °C. Cells were disrupted by freezing and thawing three times, obtaining the homogenate,[29] and cell lysate was stored at −80 °C. Total protein concentration was measured according to Bradford.[30]

cAMP-PDE activity was determined according to the method of Kincaid and Manganiello[27] with minor modifications. Briefly, platelet lysate (64 μg of protein/mL) was incubated with 0.5 μM cAMP and 63 nCi [^{3}H]-cAMP suspended in 30 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$; final reaction volume was 250 μL. After 5 min of incubation at 30 °C, the reaction was stopped with 0.1 N HCl. Samples were then incubated for a further 4 min at 70 °C with AMP (5 mM) and cAMP (5 mM), and the pH was adjusted to 7 on ice with 0.1 N NaOH. Samples were then added with 50 μL of nucleotidase from *Crotalus adamanteus* snake venom (1 mg/mL in Tris-HCl 0.1 M, pH 8.0) and incubated for 20 min at 37 °C. The reaction was stopped with 50 μL of 200 mM NaEDTA containing 5 mM adenosine. The nucleoside formed during the incubation was separated from the unreacted substrate by DEAE-Sephadex A25 column chromatography. The eluted [^{3}H]-adenosine was counted in a β-scintillation counter. Compound **5** and sildenafil were tested in a range of 1−250 μM, and IC_{50} values calculated using Graph Pad Prism 4 for sigmoidal curves. Inhibition (%) by aminophylline (100 μM) used as reference compound was 74.5 ± 1.3 (mean ± SD, n = 11). Each result is the mean ± SD of three experiments in triplicate.

Cytotoxicity Assay. Cellular toxicity was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) colorimetric assay.[31] Human skin fibroblasts were treated with increasing concentrations (0.25−100 μM) of **5** for 24 h in DMEM-F12 supplemented with 10% heat-inactivated FBS, 1% penicillin, and 1% L-glutamine. The medium was removed, and cells were incubated with a solution containing MTT 0.5 mg/mL in PBS at 37 °C for 3 h. The MTT solution was removed, the formazan was extracted with 2-pro-

panol/DMSO (9:1; 500 μL/well) for 15 min at 37 °C, and aliquots of 100 μL were read on a plate reader (Bio-Rad Laboratories) at 560 nm.

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Supporting Information Available: Figures showing the HPLC traces of the extracts under study and the effects of plant extracts and pure compounds on the inhibition of PDE5A1. This information is available free of charge via the Internet at http://pubs.acs.org.

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